Molecular Characterization and Evaluation of Probiotic Potential of *Lactobacillus rhamnosus* CW48isolated from Cow Milk

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Abstract: *Lactobacillus* strains are inhabitant of versatile environmental niche and forms a major part of the probiotics, intestinal microflora and used in the production of fermented dairy products. The objective of the present study was to evaluate the probiotic potential of *Lactobacillus rhamnosus* CW48 isolated from cow milk. Properties like antibacterial activity, bile tolerance and antibiotic resistance forms the basis for an organism to be a probiotic organism. *L.rhamnosus* CW48 showed antagonistic activity in cell free supernatant (CSF) against gram-positive organisms like *Bacillus subtilis* and *Bacillus cereus*, and gram-negative organisms like *Escherichia coli* and *Pseudomonas putida*. In neutralized CSF antibacterial activity was seen against all test organisms except *P.putida*. When the neutralized CFS was treated with enzyme protease (20mg/ml), zone of inhibition disappeared, confirming the role of bacteriocin protein in antibacterial activity. *L.rhamnosus* CW48 tolerated bile salt-oxgall up to the concentration of 0.3% (w/v) and also showed resistance to various antibiotics used in present study. *L.rhamnosus* CW48 possess antibacterial activity, bile tolerance and antibiotic resistance. The isolate can be used as a potential probiotic organism for the benefit of human population by improving health services.

Keywords: - **antibacterial activity, antibiotics, bacteriocin, bile tolerance, cow milk, LAB, *Lactobacillus rhamnosus*, probiotic**

INTRODUCTION:

Lactic acid bacteria (LAB) are common habitant of animals as well as human gastrointestinal tract. They are able to produce lactic acid and are prominently present in milk and milk products such as fermented milk, yogurt and cheeses etc. Chemical bio-preservatives causes harmful side effects and allergic reactions, beneficial and non-pathogenic genera such as lactic acid bacteria (*Lactobacillus, Lactococcus, Streptococcus, Pediococcus* and *Leuconostoc)* are now widely used in food industry as biopreservative agents (Arokiyamary *et a*l., 2011).Lactobacilli are one of the most important types of known probiotic organisms. According to the Food and Agriculture Organization (FAO), probiotic bacteria are defined as" live microorganisms that when administered in adequate amounts confers health benefit on the host" (FAO, 2001). Lactobacilli produce various metabolic by-products that play an important role in controlling the undesirable microflora in the gut. They inhibit the growth of pathogenic bacteria in different ecosystems by the production of antimicrobial substances- organic acids such as lactic acid and acetic acid, hydrogen peroxide and bacteriocins (Jin *et al*., 1996).Lactobacilli have the ability to produce desirable changes in taste, flavor and texture of fermented foods. They are considered as ' GRAS' (generally recognized as safe) organisms due to their ubiquitous appearance in food and their contribution to the health microflora of human mucosal surfaces. Bacteriocins are proteinaceous bio-molecules, synthesized by ribosomes. Also know as antimicrobial peptides (AMPs) produced by bacteria that target other bacteria, either within the same species or across different genera. A. Gratia in 1925 first discovered bacteriocins. It is well established that LAB produce high amounts of bacteriocins. In this paper, we will determine the probiotic potential of lactobacilli isolated from cow milk. Probiotic properties like antibacterial activity of lactobacilli due to the presence of bacteriocin, bile salt tolerance and antibiotic resistance property will be evaluated.

MATERIALS AND METHODS

i) Sampling, Isolation and purification of lactic acid bacteria: Cow milk sample was collected from local dairy of Udaipur city, Rajasthan, India. Lactobacilli were isolated using selective medium MRS agar, by standard pour plate method. The plates were incubated at 37°C for 48 h. Isolated colonies recovered from the plates, were further transferred into sterilized MRS broth medium. These inoculated culture tubes were incubated for 24 h at 37°C. After turbidity was observed, successive streaking was done to purify the isolates.

ii) Cultural and morphological characterization: Selected colonies were screened on the basis of cultural characterization-based on the colony characteristics and the morphology was studied by using the technique Gram staining.

iii) Antibacterial Activity: The antagonistic activity was evaluated by using well diffusion method described by Ogunbanwo *et al*,. 2003 against four bacterial strains, among them two were gram-positive and two were gram-negative bacteria *Bacillus*

subtilis(MTCC 121), *Bacillus cereus* (MTCC 430) and *Escherichia coli* (MTCC 443),*Pseudomonas putida* (MTCC 1194) respectively. The antibacterial activity was determined in CSF without neutralization and CSF neutralized with 1N NaOH. Enzyme (protease-20mg/ml) treatment was done to determine the role of bacteriocin in the antibacterial activity, for this mixing of enzyme and neutralized supernatant in equal amount was done and the treatment was maintained in incubated at 37°C for 2-3 hours. Zone of inhibition extending laterally was measured with the help of scale. The diameter of the zone of inhibition extending laterally around the well was measured with the help of scale.

v) Biochemical and Molecular characterization of isolates: The isolates were characterized on the basis of cultural, morphological, biochemical and molecular basis. Biochemical characterization was based on catalase test, growth on MRS agar supplemented with bromocresol purple (BCP) dye, esculin hydrolysis, arginine hydrolysis, nitrate reduction, litmus milk coagulation, gas production, growth on different temperatures, indole production, citrate utilization, gelatin hydrolysis and carbohydrate fermentation pattern using different sugar discs like mannose, fructose, sorbitol, lactose, maltose, mellibose, galactose, raffinose, sucrose, xylose, trehalose and rhamnose.

Molecular characterization of the isolates is based on genomic DNA isolation, PCR based enhancement of products and sequencing of amplified products. The genomic DNA was isolated by Pospeich and Neumann's method (1995). Isolates were subjected to PCR using semiuniversal *Lactobacillus* genus specific primer Lb1 (5'- AGAGTTTGATCATGGCTCAG-3') and Lb2 (5'-CGGTATTAGCATCTGTTTCC-3') based on variable loop of 16S rDNA sequence designed by Klijn *et al*., 1991. For sequencing the amplified products were sent to SciGenome Labs Pvt Ltd. Kochi, India. The partially sequenced data obtained were analyzed by BLAST and submitted to EMBL-EBI database.

vi)Bile salt tolerance: The bile tolerance of isolates was evaluated as per the method suggested by Sirilun *et al*., 2010. MRS agar medium supplemented with oxgall at different concentrations such as 0.1, 0.2, 0.3, 0.4 and 0.5% were used. The medium was streak and incubated at 37°C for 24h to 72h.

vii)Antibiotic resistance: The antibiotic resistance of lactobacilli was evaluated using disc diffusion method (Bauer *et al*., 1966). The antibiotic discs (Himedia) used were of ampicillin (10mcg/disc), tetracycline (30mcg/disc), kanamycin (30mcg/disc), streptomycin (25µg/disc), penicillin (10units/disc), rifampicin (30µg/disc), amikacin (10mcg/disc), polymyxin (300unit/disc), cefixime (5mcg/disc), vancomycin (30mcg/disc), gentamycin (30mcg/disc) and trimethoprim (5mcg/disc). The plates were incubated at 37°C for 24 h. The diameter of the inhibition zone was measured with a scale including the disc area.

RESULTS:

A total of 15isolates were recovered on MRS agar medium using standard pour plate method from cow milk sample. Purification of these 15 isolated was done by continuous streaking and transferring pure colonies into fresh medium. On the basis of cultural and morphological characterization among these 15 isolates, 8 isolates appeared to be rod shaped and gram-positive and colonies of all the selected isolates appeared as pin pointed, white colored, with entire margin and convex elevation. Rest of the isolates (7) were found to be cocci shapedand gram-negative.

A total of 5 out of 8 lactobacilli isolates showed antibacterial activity in CFS (cell free supernatants) without neutralization against all four test organisms namely *B.cereus*, *B.subtilis*, *E.coli* and *P. putida*. These 5 lactobacilli isolates were further tested for antibacterial activity using neutralized CFS with 1N NaOH. A total of 2 lactobacilli isolates namely (isolates CW48 and CW58) out of 5 showed antibacterial activity against three 3 test organisms (*B.cereus*, *B.subtilis* and *E.coli*). The neutralized CFS of these 2 lactobacilli isolates were subjected to enzyme (protease) treatment and were further tested for antagonistic activity due to the production of bacteriocin against three test bacteria. The inhibition zone disappeared in only one isolate namely CW48, indicating bacteriocin role in antagonistic activity of the isolate. Antibacterial activity of isolate CW48 against *B. subtilis*is shown in table1 and figure 1.

Table 1 Antibacterial activity of *Lactobacillus rhamnosus* CW48 without neutralization, with neutralization and after enzyme (protease) treatment against test organisms

S. no	Test organism	Diameter of inhibition zone (cm)		
		Supernatant without NaOH Mean ± S.D.	Supernatant with NaOH Mean ± S.D.	Neutralized supernatant with Protease Mean ± S.D.
1.	*E. coli*	1.46±0.12	1.2±0.08	NZ (0)
2.	*B. cereus*	1.36±0.12	1.16±0.09	NZ (0)
3.	*B. subtilis*	1.43±0.16	1.13±0.12	NZ (0)

4.	*P.putida*	1.50±0.08	NZ (0)	NZ (0)

NZ (0)= no zone of inhibition

Figure 1 Antibacterial activity of *Lactobacillus rhamnosus* CW48 against *Bacillus subtilis*
A= CW 58 crude cell free supernatant without neutralization
B= CW 58 crude cell free supernatant neutralization with 1N NaOH
C= CW 48 crude cell free supernatant without neutralization
D= CW 48 crude cell free supernatant neutralization with 1N NaOH

Isolate CW48 was further characterized using biochemical tests and 16S rRNA partial gene sequencing. The isolate was found to produce negative for catalase activity. Isolate CW48 showed yellow colored colonies on MRS medium supplemented with BCP dye and was able to hydrolyze esculin. Isolate CW48produced negative reaction for nitrate reduction, gelatin hydrolysis, gas production from glucose and indole production. Isolate CW48also showed negative result for arginine hydrolysis and positive reaction for litmus milk coagulation. CW48 showed growth on both 15°C and 45°C incubation temperatures.Isolate CW48 showed varied response for carbohydrate fermentation reaction. It was able to fermentmannose, fructose, sorbitol, lactose, maltose, galactose, sucrose, trehalose and rhamnose. It was unable to ferment mellibiose, raffinose and xylose.

The genomic DNA isolation of the isolate CW48 was done by Pospeich and Neumann's method and was amplified by PCR using semi universal primers (Lb1 and Lb2). Isolate CW48 gave 200bp product which was further sequenced. The sequenced data obtained was analyzed by BLAST and was submitted to EMBL gene data base under the accession no. LT797532. Phylogenetic relationship was studied and tree was drawn using NCBI-BLAST neighbor- joining method, relationship between strain *L.rhamnosus*CW48 and other known sequences of *L.rhamnosus* is presented in figure 2.

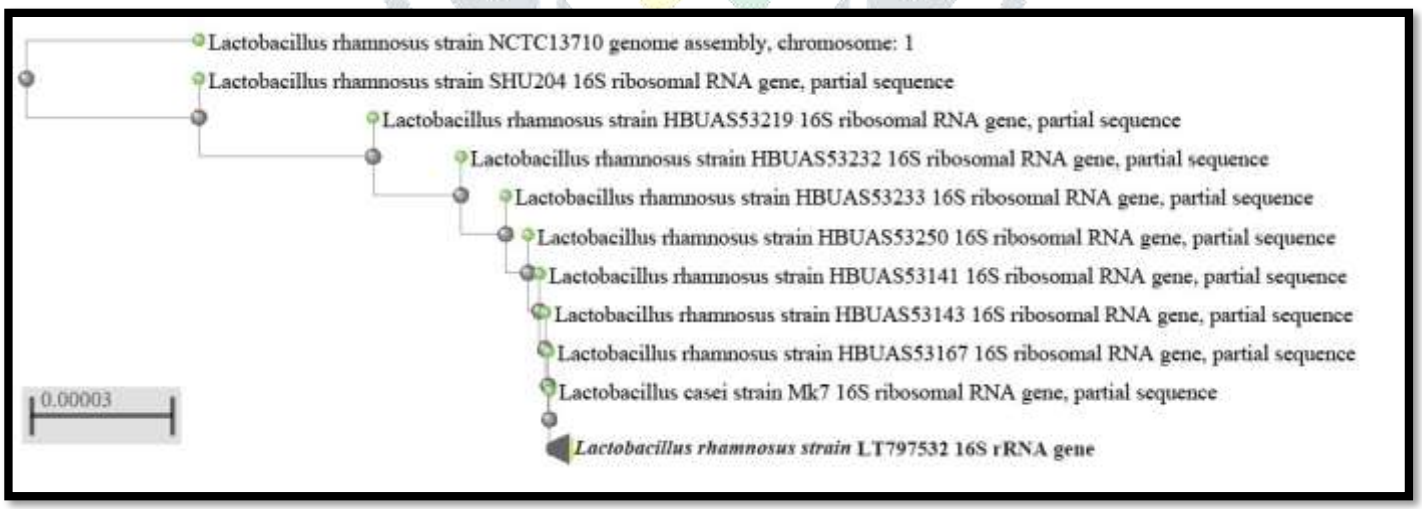

Figure 2: Phylogenetic tree of *L.rhamnosus* CW48 based on 16S rRNA sequencing

Lactobacillus rhamnosus CW48 showed varied degree of growth in MRS agar supplemented with different concentrations of bile salt-oxgall (0.1, 0.2, 0.3, 0.4 and 0.5%). The isolate was able to tolerated two concentrations (0.1 and 0.2%) of oxgall, as it showed maximum growth after 72h of incubation. The isolate also tolerated 0.3% of oxgall and showed comparatively less growth even after 72h of incubation. Isolate was unable to tolerate 0.4% and 0.5% of oxgall and no growth appeared even after 72h of incubation. Data presenting the tolerance of *L.rhamnosus*CW48 to different concentrations of oxgall is shown in table 2.

Table-2 Bile tolerance of *Lactobacillus rhamnosus* CW48

S. no	Concentration of oxgall (%)	Growth of *Lactobacillus rhamnosus* CW48		
		Incubation time (hours)		
		24 h	48h	72h
1.	0.1	+	++	++
2.	0.2	+	++	++
3.	0.3	-	+	+
4.	0.4	-	-	-
5.	0.5	-	-	-

+ = less growth; ++= maximum growth; - = No growth

Antibiotic resistance of *L.rhamnosus*CW48 was evaluated against 12 different antibiotics. The isolate showed resistance to five antibiotics (namely kanamycin, cefixime, polymyxin B, trimethoprim and vancomycin) and was found sensitive to seven antibiotics (namely ampicillin, tetracycline, streptomycin, gentamycin, penicillin, rifampicin, and amikacin).

DISCUSSION:

Present study focuses on the isolation of lactobacilli from cow milk, study of their antibacterial activity due to the action of bacteriocin protein, their characterization and determination of their probiotic potential using properties such as bile salt (oxgall)tolerance and antibiotic resistance of selected bacteriocin producing strain.

The antibacterial activity of lactic acid bacteria is extensively studied and reported worldwide (Niel *et al*., 2002). Antibacterial activity is one of the most essential properties of an organism to become a functional probiotic. The production of antimicrobial compounds provide them edge to their survival, proliferation in gastro intestinal (GI) tract and also helps in eradicating pathogenic bacteria.LAB are known to produce various antimicrobial components which can be classified as: (i) Low molecular mass compounds such as diacetyl, carbon dioxide and H_2O_2 (ii) High molecular mass compounds like bacteriocin (Ammor *et al*., 2006 ; Mobolaji & Wuraola, 2011).

In present investigation, CW48 showed considerable antagonistic activity in CFS (cell free supernatant) without neutralization and neutralized CFS against pathogenic test bacteria, confirming the role of both low molecular mass and high molecular mass compounds during inhibition of pathogenic bacteria. Mobolaji & Wuraola, (2011) studied and confirmed the primary role of lactic acid in antimicrobial effects exerted by LAB. Results of present study are in agreement with Mobolaji & Wuraola, (2011). The CFS of CW48 without any neutralization showed stronger inhibition as compared to neutralized supernatant, similar finding were reported by Sirilun *et al*., 2010.

Antagonistic activity present in cell free supernatant even after neutralization indicates the presence of bacteriocin in test sample. Similar inhibitory activity has been reported against a number of other bacteria (Ogunbanwo*et al*., 2003). To confirm the antagonistic activity produced by crude bacteriocin, it was treated with proteolytic enzyme. There was no zone of inhibition observed after enzyme treatment. This indicates the proteinaceous nature of antimicrobial component present. Same methodology was adopted by Schved*et al*., 1993 and the results are in agreement to present investigation's results.

As per FAO/WHO one of the most widely used property for screening probiotic properties of potential strain is *in vitro* testing for bile salt tolerance, so that organism can survive in small intestine. *Lactobacillus rhamnosus* CW48 exhibited moderate range of bile salt tolerance up to 0.3% ox gall after 72 h of incubation. The normal level of bile salt in the intestine is around 0.3% (Moura & Eddine, 2006). Mathara *et al*., 2008 proposed that the efficient probiotic bacteria should be able to grow in bile concentration ranging from 0.1 to 0.5%. The results of present investigation are in accordance with the findings of Moura & Eddine, 2006 and Mathara*et al*., 2008.

Another important probiotic property for lactobacilli is to be able to resist broad spectrum antibiotics. So that lactobacilli can remain viable in gut and impart its beneficial effects to its host. *L. rhamnosus* CW48 was found to resist the effect of antibiotic kanamycin, a translation inhibitor. This inhibition of lactobacilli towards kanamycin is frequent. The finding of present investigation is similar with the findings (Danielsen & Wind, 2003; Coppola *et al*., 2005; Zhou *et al*., 2005).

Cephalosporins like cefixime are class of β-lactam antibiotics originally derived from fungus *Acremonium*. These are bactericidal drugs and disrupt the synthesis of peptidoglycan layer forming the bacterial cell wall. *L. rhamnosus* CW48 showed no inhibition for cefixime. Ammor *et al*. 2007 reported that lactobacilli shows resistance against cephalosporins like cefixime. Results are in accordance with above mentioned findings.

L.rhamnosus CW48 showed high level of sensitivity against protein synthesis inhibitor tetracycline. Similar findings were reported by Ammor*et al*., 2007 and Belletti *et al*.,2014. Some researchers suggested that *tet*(M) gene is responsible for

tetracycline resistance in LAB (Danielsen, 2002; Gevers *et al*., 2003), in this regards the results of present study are in contrary to the above mentioned findings.

L. rhamnosus CW48 showed resistant to vancomycin, cell wall synthesis inhibitor. D'Aimmo *et al*., 2007 and Tynkknen *et al*.,1998 proved that lactobacilli are resistant to cell wall inhibitors like vancomycin. Results explained of the present study showed similarity with the previous studies. Tynkken*et al*., 1998 this kind of resistance is intrinsic which is chromosomally encoded and non-transmissible. This may be the possible explanation to the results presented in present investigation.

CONCLUSION

Lactobacillus rhamnosus CW48 showed remarkable antagonistic activity against pathogenic bacteria in both the crude supernatant and neutralized supernatant. CW48 can tolerate bile (ox gall) and was found to be resistance against varied range of antibiotics. This strain can act as a potential probiotic organism and can also be used as starter culture for the production of fermented food products for providing beneficial effects to human health.

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